The dog as model for chemotherapy of the Chagas' disease

Paulo Marcos da Matta Guedes [a], Vanja Maria Veloso [a], Washington Luiz Tafuri [a], Lúcia Maria da Cunha Galvão [b], Cláudia Martins Carneiro [c], Marta de Lana [c], Egler Chiari [b], Killarney Ataide Soares [a], Maria Terezinha Bahia [a],[*]

[a] *Departamento de Ciências Biológicas, Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto, UFOP, Morro do Cruzeiro, CEP 35400-000, Ouro Preto, MG, Brazil*
[b] *Departamento de Parasitologia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil*
[c] *Departamento de Análises Clínicas, Universidade Federal de Ouro Preto, Ouro Preto, MG, Brazil*

Abstract

In the present study, we investigated the role of dogs as experimental models for acute and chronic phases of Chagas' disease, before and after therapeutic treatments. Dogs were infected with *Trypanosoma cruzi* strains of different susceptibilities to benznidazole (Bz) and treated with the same therapeutic scheme as used for human chagasic. The treatment with Bz was able to prevent death and induced parasitological cure in 62.5% (acute phase) and 38.7% (chronic recent phase) of the tested animals. These results were similar to those reported in clinical trials for treated human patients (cured and uncured) in both phases of the disease. We also showed that parasitologic and serologic tests for monitoring the cure were similar to those obtained for human trials. In addition, Polymerase chain reaction showed the highest sensitivity when compared with hemoculture as an indicator of parasite clearance. In conclusion, the proposed experimental model should be relevant for chemotherapy studies for the control of Chagas' disease. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Canine experimental model; Chagas' disease; *Trypanosoma cruzi*; Chemotherapy

1. Introduction

Chagas' disease is endemic in South American countries, where it is estimated 16–18 million persons are affected and 50 000 deaths occur annually (WHO, 1993). Although great progress has been made, recently, in the control of the vector and in the transfusional transmission of the disease, the specific treatment of infected individuals remains unsolved.

Drugs such as nitrofurans and nitroimidazoles have been used with variable degree of therapeutic

* Corresponding author. Tel.: +55-31-3559-1690
E-mail address: mtbahia@nupeb.ufop.br (M.T. Bahia).

success in the acute phase of the disease, and in children in the chronic phase (Shikanai-Yasuda et al., 1990; Andrade et al., 1996; Sosa Estani et al., 1998). Unfortunately, there are no drugs that are highly successful at treating in chronic phases of the disease (Ferreira, 1990). The fact of the matter is the eradication of *Trypanosoma cruzi* in chagasic patients is of primary importance to prevent the development of the disease and avert its irreversible long-term consequences (Viotti et al., 1994; Andrade et al., 1991).

The evaluation of therapeutic effectiveness of chronic chagasic patients is very difficult due to slow development of the disease, which requires long patient follow-up periods. Moreover, available parasitological methods present low sensitivity, and anti-*T. cruzi* antibodies persist for many years post-treatment in a large percentage of the patients (Galvão et al., 1993). Lytic antibodies are detected by the complement mediated lysis test, which is used to evaluate treatment efficacy (Krettli et al., 1984; Galvão et al., 1993).

The establishment of experimental models that reflect the human disease and studies for specific treatment of Chagas' disease are necessary. Experimentally infected dogs develop acute and chronic phases of chagasic infection that are comparable to clinical signs of the human disease (Andrade and Andrade, 1980; Andrade, 1984; Lana et al., 1992).

In this report, a specific treatment was evaluated in a canine experimental model system for both, the acute (acute model) and chronic (chronic model) phases of Chagas' disease. For the acute model, 50% of the animals were infected with *T. cruzi* sensitive to benznidazole (Bz), and 50% with partially resistant and resistant strains. This model takes into consideration the population proportions of Bz sensitive (58.6%), partially resistant and resistant strains (42.3%) and the cure index of 60–70% in the acute phase of Chagas' disease as reported by different researchers (Andrade et al., 1985, 1992; Filardi and Brener, 1987; Galvão et al., 1993; Toledo et al., 1997). For the chronic model, all of the dogs were infected with *T. cruzi* strain Berenice-78, since it is still not clear the importance of the parasites

population characteristics on the cure index of this phase.

2. Materials and methods

2.1. Trypanosoma cruzi strains

T. cruzi strains used in this study were: Colombian, *T. cruzi* I (Federici et al., 1964), a resistant strain to Bz (Veloso et al., 2001)Y, *T. cruzi* (Silva and Nussenzweig, 1953), a partially resistant strain to Bz (Filardi and Brener, 1987), and Berenice-78, *T. cruzi* II (Lana and Chiari, 1986), which is susceptible to Bz (Veloso et al., 2001).

2.2. Experimental animals and infection

Thirty-two male and female 3 months old mongrel dogs from the kennel of the Federal University of Ouro Preto, MG, Brazil were fed a commercial ration, and water was available ad libitum. Before the study, the animals were treated with anti-helminthes and immunized against infectious diseases. For the acute model, the animals were inoculated with 2000 blood trypomastigotes per kg of bodyweight via peritoneal, of *T. cruzi* Berenice-78 (10 dogs), Y (6 dogs) and Colombian (6 dogs) strains. For the chronic model, 10 animals were inoculated with Berenice-78 strain. Two untreated dogs were used as the positive control group.

2.3. Treatment schedule

The treatment schedule was 7 mg of Bz per kg bodyweight administered orally in two doses daily for 45 days. In the acute model, the animals were treated 12–22 days after *T. cruzi* detection, while in the chronic model they were treated 100 days after inoculation. The animals were considered cured when parasitologic (fresh blood examination, hemoculture), molecular (polymerase chain reaction, PCR) and serological methods (enzyme-linked immunosorbent assay, ELISA and complement mediated lysis, CoML) were all negative.

2.4. Parameters evaluated

2.4.1. Parasitaemia and mortality

The parasitaemia of the animals was examined from the 10th day of infection until the parasites could no longer be detected by collecting fresh blood from the marginal ear vein. Mortality rate was expressed as a cumulative percentage of animals that died.

2.4.2. Hemoculture

The hemoculture technique (Chiari et al., 1989) was performed before treatment, and at 1 and 6 months post-treatment for acute and chronic models. Hemocultures were examined monthly up to 120 days for detecting *T. cruzi* parasites.

2.4.3. Polymerase chain reaction assay

Ten milliliters of blood were collected from acute and chronic animals 1 and 6 months post-treatment. The samples were immediately mixed with an equal volume of 6 M guanidine HCl/0.2 M EDTA solution (Ávila et al., 1991), maintained at room temperature for 1 week and boiled for 15 min to break the minicircles (Britto et al., 1993). Three DNA extractions were performed according to Wincker et al. (1994), but using 40 µg of Glycogen (Boehringer Mannheim) to precipitate the DNA. PCR conditions were the same described by Gomes et al. (1998), but 20 pmol of S35 (5′-AAATAATGTACGGG(T/G)GAGATG-CATGA-3′) and S36 (5′-GGGTTCGATTGG-GGTTGGTGT-3′) primers were used (Ávila et al., 1990). Briefly, 2 µl of blood DNA template was added to 10 mM Tris–HCl (pH 9.0), 75 mM KCl, 3.5 mM MgCl$_2$, 0.1% Triton X-100, 0.2 mM of each dATP, dCTP, dGTP and dTTP (Sigma Chemical Co.), 1.0 unit of Taq DNA polimerase (Promega), and water in a 20 µl reaction volume. The reaction mixtures were overlaid with 30 µl of mineral oil and subjected to 35 cycles of amplification in a Research Programmable Thermal Controller (MiniCycler TM). The temperature profile was 95 °C for 5 min for denaturation, two cycles with annealing at 30 °C for 2 min, followed by 33 cycles with the annealing temperature increased to 40 °C and a final extension at 72 °C for 5 min. Five microliters of PCR products were analyzed by electrophoresis on a 6% polyacrylamide gel and visualized by silver staining (Santos et al., 1993).

2.4.4. Serological profile

Serum samples were collected from the blood of acute infected dogs before and monthly after the inoculation for 6 months and of those chronically infected up to 3 years post-treatment. The serum samples were stored at −20 °C and ELISA tests were performed according to Voller et al. (1976). *T. cruzi* Y strain obtained from acellular culture in LIT medium (ELISA–epimastigote) and from VERO cellular culture (ELISA–trypomastigote) were used as antigens. The sera were tested in parallel using both antigens and peroxidase conjugated goat anti-dog IgG (Sigma Chemical Co.). The cut-off was determined using the absorbance mean of 10 uninfected animals plus two standard deviations.

2.4.5. Complement mediated lysis

The CoML assay with *T. cruzi* Y strain trypomastigotes from tissue culture were performed before and 6 months post-treatment in the acute model, and for the chronic model before, 9 and 24 months post-treatment. This test was performed as described by Krettli et al. (1979) and modified by Galvão et al. (1993). Briefly, trypomastigotes forms from VERO cellular culture were concentrated to 6×10^6 parasites/ml. An aliquot of 50 µl of a suspension of these trypomastigotes plus 50 µl of the test serum (1:2 and 1:4 dilutions) were incubated at 37 °C for 30 min and then placed on ice. Fresh human serum from healthy volunteers was added as a source of complement and the number of living trypomastigotes was counted on a hemocytometer. Tubes were then reincubated in the same conditions for 45 min to determine the percentage lysis. Samples were examined at microscopy, and the number of motile intact trypomastigotes were counted again and compared to the number of lysed parasites. This assay was considered positive when lysis was greater than 20%. Sera used as negative control for the serological tests were also used as control for the CoML reaction.

3. Results

3.1. Side-effects and toxicity

Bz was well tolerated by the dogs and no side effects were observed during the study.

3.1.1. Acute model

The parasitaemia was suppressed from the first to the third day post-treatment in all treated animals. However, three (18.75%) and five (31.25%) out of 16 treated animals showed positive hemoculture and PCR results, respectively, in at least one test (Table 1). All untreated control animals were positive for hemoculture and PCR.

High levels of *T. cruzi* specific IgG antibodies were detected around 20 days after inoculation in sera of the untreated control group (Fig. 1A and B). Among treated animals, the antibody levels were below of the cut-off limits 20 weeks post-treatment in 11 animals, seven of them infected with Berenice-78 and four with Y strains (ELISA–epimastigote). Five animals showed antibody levels similar to untreated controls, one infected with Berenice-78 and four with Colombian strain (Fig. 1A). ELISA–trypomastigotes assays showed antibody levels higher than the cut-off limits in all treated animals (Fig. 1B).

Lytic antibodies were detected in sera of 75% of infected dogs between the 12th and the 21st day (Fig. 2A) and remained positive in the control group. Among the treated animals, the CoML was persistently positive 6 months post-treatment in five dogs, one of them infected with Berenice-78 and four with the Colombian strain (Fig. 2A). All animals that had negative parasitologic, PCR and ELISA–epimastigote tests also had negative CoML 6 months post-treatment (Fig. 2A).

Based on hemoculture, PCR and serologic (ELISA–epimastigote and CoML) tests, 68.75% (11/16) of the treated animals were considered cured.

3.2. Chronic model

Parasitologic, molecular (Table 2 and Fig. 3) and serologic (Fig. 2B, Fig. 4A and B) tests were positive in all infected animals with Berenice-78

strain before treatment. On the other hand, at least one test of hemoculture and PCR remained positive in 12.5% and 62.5% of the animals, respectively, post-treatment. Both tests were positive in the untreated animals (Table 2 and Fig. 3).

The serologic pattern was clearly different between treated and controls animals in ELISA–epimastigote test (Fig. 4A). Based on parasitologic and PCR evaluations, the animals were divided into three groups: (1) untreated controls; (2) treated with positive parasitologic and PCR tests and (3) treated with negative parasitologic and PCR tests. A slow decrease of absorbance levels was observed in the group 1 during the experimental period (Fig. 4A). On the other hand, groups 2 and 3 showed an abrupt decreasing in the absorbance levels after beginning the Bz treatment. However, the absorbance levels of the group 2 were always slightly higher than group 3 (Fig. 4A), while three animals of the group 3 showed a negative sero-conversion at the 37th, 72nd and 113th weeks post-treatment. The treatment did not induce changes in serologic patterns, which were similar to the control group when ELISA–trypomastigote testing was used (Fig. 4B).

Lytic antibodies persisted in higher levels for 9 months after treatment, even in sera of animals that had negative parasitologic, PCR and ELISA–epimastigote tests (Fig. 2B). Three animals with negative parasitologic, PCR and ELISA–epimastigote tests switched from CoML positive to negative 24 months post-treatment. In the untreated control and treated animals with positive parasitologic, PCR and serologic tests, the CoML was persistently positive (Fig. 2B).

On the face of parasitologic, PCR and serologic results, 37.5% (3/8) of treated animals were considered cured.

4. Discussion

A major goal on Chagas' disease research is the development from specific drug to eliminate *T. cruzi* from acutely or chronically infected individuals. Moreover, the establishment of routine methods to discriminate among infected and cured patients and the implementation of studies to
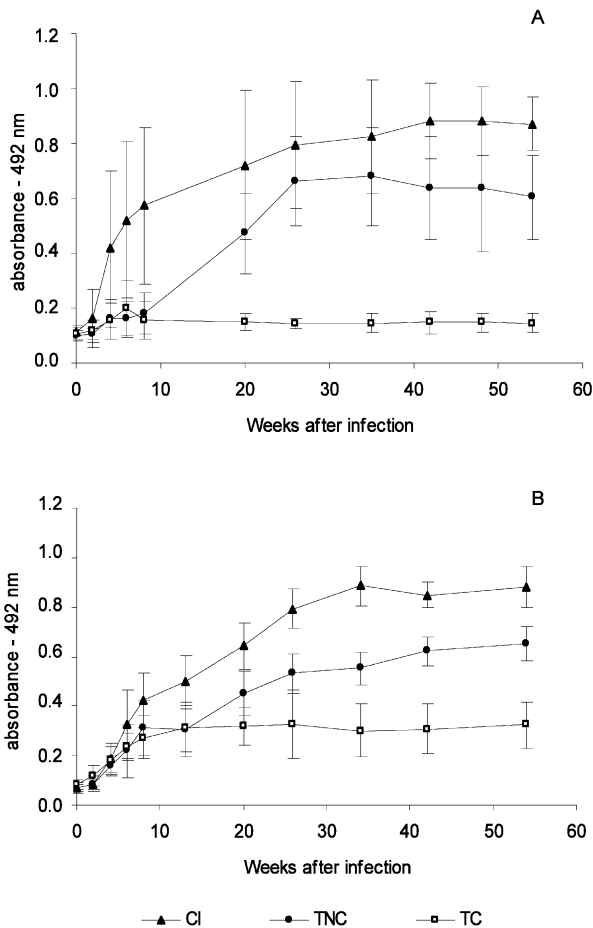
Fig. 1. *T. cruzi* specific IgG antibodies in sera of infected control group (CI) and dogs treated in the acute phase of the infection with 7 mg of benznidazole per kg bodyweight, divided in two daily doses, for 45 days. TC cured and TNC noncured animals ((A) ELISA–epimastigote; (B) ELISA–trypomastigote).
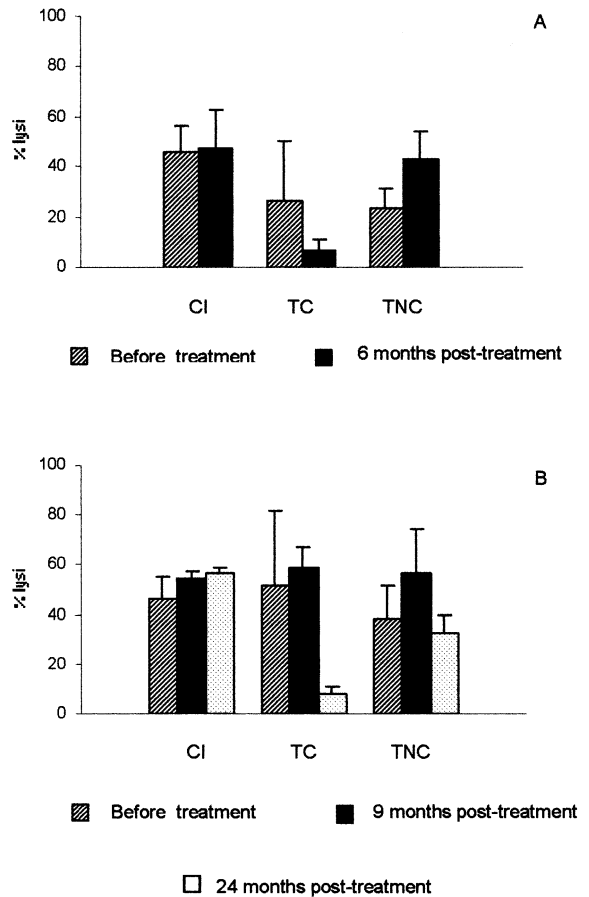

Fig. 2. Percentage of lytic antibodies in sera of the infected control group (CI), treated and cured (TC) and treated uncured animals (TNC). (A) Animals treated in the acute phase and (B) animals treated in chronic phase of the disease. All animals were treated with 7 mg of benznidazole per kg bodyweight, divided in two daily doses, for 45 days.

establish experimental models to evaluate potential therapeutic agents for the control of this disease are important. In this work, we reported the relative therapeutic efficacy of Bz against *T. cruzi* in a canine model for acute and chronic phases of the disease.

Parasitological and serological tests were negative in 67.5 and 37.5% of treated animals during the acute and recent chronic phases, respectively. Most clinical trials have reported cure rates of 60–70% for the acute phase of the infection (Andrade et al., 1992; Galvão et al., 1993; Bahia-Oliveira et al., 2000). Cure rates of only 10–17% have been reported for patients in the chronic phases of Chagas' disease (Ferreira, 1990; Galvão et al., 1993; Braga et al., 2000). However, recent publications have described an efficacy from 55.8 to 62% in children who received Bz treatment (Andrade et al., 1996; Sosa Estani et al., 1998). Similarly, the time of infection could be a decisive factor in the therapeutic success, since in humans or in our model, the cure index was higher than those obtained in adult patients (Cançado, 1999). Another factor that may interfere with the therapeutic effectiveness is the time of drug administration because in clinical trials, where the

Table 1
Parasitologic and molecular tests in blood of infected dogs with *T. cruzi* Berenice-78, Y and Colombian strains and treated in acute phase with 7 mg of benznidazol for 45 days

Groups Treated	Survived animals/total of animals	Positive Hc/No. tested		Positive PCR/No. tested		Positive parasitological tests/total of animals
		Months after treatment				
		1st	6th	1st	6th	
Treated						
Berenice-78	8/8	1/8	0/8	1/8	1/8	1/8 (12.5%)
Colombian	4/4	1/4	1/4	4/4	4/4	4/4 (100%)
Y	4/4	0/4	0/4	0/4	0/4	0/4 (0%)
Untreated	4/6	2/4	3/4	4/4	4/4	4/4 (100%)

No., Number; Hc, Hemoculture.

Table 2
Parasitologic and molecular tests in blood of infected dogs with *T. cruzi* Berenice-78 strain and treated in chronic phase with 7 mg of benznidazole for 45 days

Groups	Survived animals/total animals	Positive Hc/No. tested			Positive PCR/No. tested			Positive tests post-treatment/total animals
		Months after treatment						
		Before	1st	6th	Before	1st	6th	
Treated	8/8	6/8	0/8	1/8	8/8	5/8	5/8	5/8 (62.5%)
Untreated	2/2	2/2	1/2	1/2	2/2	2/2	2/2	2/2 (100%)

No., Number; Hc, Hemoculture.

treatment was administered for 30 days in chronic chagasic patients, the cure rate was 0–3% (Braga et al., 2000; Fabbro de Suasnábar et al., 2000). Therefore, the cure rate (37.5%) of the treated animals in recent chronic phase could be related to the time of treatment (45 days).

The cure indexes of infected animals with *T. cruzi* Berenice-78 and Colombian strains were similar those obtained with the murine model (Veloso et al., 2001; Filardi and Brener, 1987). On the other hand, all of the dogs infected with *T. cruzi* Y strain and treated in the acute phase were cured. This is interesting because this strain was partially resistant to Bz, in experimentally infected mice that showed a cure rate of 47% (Filardi and Brener, 1987). The susceptibility to Bz and Nifurtimox compounds can be related to genetics characteristics of *T. cruzi* populations (Revollo et
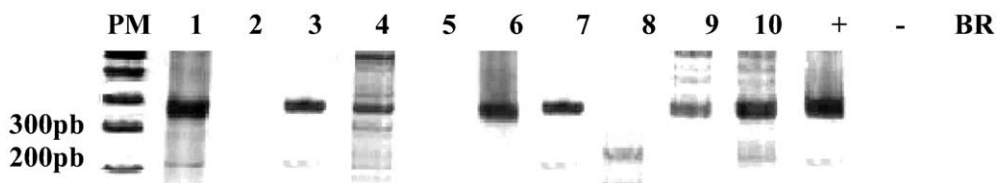

Fig. 3. Amplified PCR products ≈ 330 base pairs (bp) on 6% polyacrilamide gel, silver stained. MW, molecular weight 100 bp ladder; lanes 2, 5 and 8 treated and cured; lanes 1, 3, 4, 6 and 7 treated and noncured, lanes 9 and 10 are infected controls; +, infected controls; −, uninfected controls, and BR is an internal control without DNA.
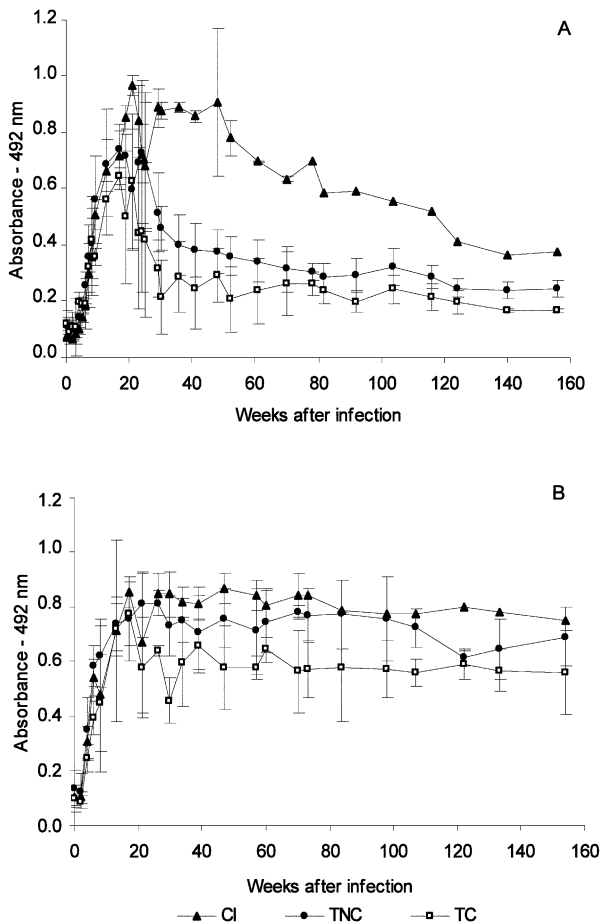
Fig. 4. *T. cruzi* specific IgG antibodies in sera of infected control group (CI) and treated dogs in the chronic phase of infection with 7 mg of benznidazole per kg bodyweight for 45 days. (TC) treated cured and (TNC) treated noncured animals ((A) ELISA–epimastigote; (B) ELISA–trypomastigote).

al., 1998; Toledo et al., 2000). This data suggest that further studies to evaluate the immune responses induced by *T. cruzi* in different host species and its relationship with Chagas' disease chemotherapy are required.

A significant reduction in the number of positive hemocultures, which indicates a decrease or clearance of parasites, shows the specific antiparasitic activity to Bz. *T. cruzi* specific DNA sequences were detected, by PCR amplification, in all animals that were positive by conventional serology and CoML assays. The high sensitivity of PCR compared with xenodiagnosis and hemocul-

ture has been used previously to measure the cure rate in treated chagasic patients (Britto et al., 1995; Silveira et al., 2000). This study reinforces the use of PCR as a useful tool for monitoring parasite cure of chagasic patients submitted to a specific chemotherapy.

Treated and cured animals in the acute phase of the chagasic infection had a negative ELISA–epimastigote serum conversion in the first month post-treatment. These findings are similar to those observed in treated patients that had negative conventional serology about 12 months post-treatment (Cançado, 1999). However, different serological profiles in ELISA related to trypomastigotes or epimastigotes antigens were observed, and these differences were striking in chronically treated animals. Similar differences have been observed in treated chagasic patients in both, acute and chronic phases, when epimastigotes or trypomastigotes were used as antigens in ELISA, indirect immunofluorescence and Western blot (Umezawa et al., 1999).

For the diagnosis of Chagas' disease, epimastigotes antigens present as inconvenient the low specificity due to cross-reactions. Recently, the high sensitivity and low specificity of epimastigotes antigens, when compared with recombinant antigens, have been reported (Umezawa et al., 1999). Therefore, post-treatment evaluation with epimastigotes appeared to be more effective than the trypomastigotes antigens.

Cure assessment in chronic chagasic infection is still controversial due to the lack of sensitive and reliable criteria, and because of the persistence of anti-*T. cruzi* antibodies post-treatment. In chronic chagasic patients treated with trypanocidal drugs, a decrease in specific antibodies has been observed and could be used as an indicator of being cure (Luquetti and Rassi, 1999). In chronic treated animals model that did not show negative seroconversion, significant reduction in antibody levels compared with baseline values and control untreated animals were observed. Other authors have observed a decrease in specific antibody levels in children previously treated with trypanocidal drugs without negative seroconversion (Andrade et al., 1996; Sosa Estani et al., 1998; Fabbro de Suasnábar et al., 2000). However, antibody levels

were not substantially changed by therapeutic activity when ELISA–trypomastigote were used, thus showing the importance of full knowledge of antibodies profiles obtained from each antigen during chagasic infection and after a specific treatment. Therefore, experimental models may be important for the evaluation of new candidate antigens.

The negative seroconversion of CoML was observed between the 6th and 24th months after treatment in 76% of treated and cured chronic chagasic patients (Galvão et al., 1993). These results were similar to those reported in our study where negative seroconversion was observed in the acute model 6 months post-treatment, and between the 9th and the 24th month for the chronic model.

In conclusion, the proposed experimental model can be of great importance for further studies on chemotherapy of Chagas' disease, which includes the evaluation of new candidate compounds. The establishment of the specific therapeutic effects on the immune state and the evolution of Chagas' disease, could initiate the development of new methods (such as PCR) or criteria for evaluating the cure of parasitosis.

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